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(54) Title: COMPOSITIONS AND METHODS FOR THE INHIBITION OF MUC-5 MUCIN GENE EXPRESSION (57) Abstract The invention relates to methods for identifying inhibitors of mucin production, methods for inhibiting mucin production and methods for treating airway diseases, such as cystic fibrosis, chronic bronchitis, bronchial pneumonia and asthma. Compositions are provided for use in the method comprising reporter gene constructs which are inducible by mucomones.		

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1 COMPOSITIONS AND METHODS FOR THE INHIBITION OF MUC-5 MUCIN
2 GENE EXPRESSION

3
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6 Service (HL24136, HL43762). The U.S. Government may have rights in this invention.

7
8
9 INTRODUCTION

10 Technical Field

11 The present invention provides for methods of identifying compounds for treating
12 medical conditions related to the inappropriate production of mucin, such as
13 *Pseudomonas* infections of cystic fibrosis patients, bronchial pneumonia, chronic
14 bronchitis and bronchial asthma.

15
16 Background

17 Mucins are a family of glycoproteins secreted from epithelial cells at many body
18 surfaces, including the eyes, pancreatic ducts, gallbladder, prostate and mainly,
19 respiratory, gastrointestinal and female reproductive tracts. Mucins are responsible for
20 the viscoelastic properties of mucus. In the airways, mucin interacts with cilia to trap and
21 clear pathogens and irritants. Bacterial infection of the airway epithelium is often
22 accompanied by mucin overproduction. In addition, airway diseases such as chronic
23 bronchitis, cystic fibrosis and asthma are characterized by mucus hypersecretion.
24 Hypersecretion can overwhelm the ability of the cilia to function properly. Mucociliary
25 impairment leads to airway mucus plugging which promotes chronic infection, airflow
26 obstruction, and sometimes death.

27 Nine mucin genes are known to be expressed in man: *MUC 1*, *MUC 2*, *MUC 3*,
28 *MUC 4*, *MUC 5AC*, *MUC 5B*, *MUC 6*, *MUC 7* and *MUC 8* (Bobek, et al. (1993) *J. Biol.*
29 *Chem.* 268:20563-9; Dusseyn, et al., (1997), *J. Biol. Chem.* 272:3168-78;
30 Gendler, et al. (1991) *Am. Rev.Resp. Dis.* 144:S42-S47; Gum, et al. (1989) *J. Biol. Chem.*
31 264:6480-6487; Gum, et al. (1990) *Biochemical and Biophysical Research*

1 *Communications* 171:407-415; Lesuffleur, et al. (1995) *J. Biol. Chem.*, 270:13665-
2 13673; Meerzaman, et al. (1994) *J. Biol. Chem.* 269:12932-12939; Porchet, et al. (1991)
3 *Biochem. Biophys. Res. Comm.* 175(2):414-422; Shankar, et al. (1994) *Biochem. J.*,
4 300:295-298; Toribara, et al. (1997) *J. Biol. Chem.* 272:16398-403). Cysteine-rich
5 domains are considered to be typical of mucin sequences, having been reported in many
6 mucins including *MUC 2* (Gum, et al. (1992), *J. Biol. Chem.* 267:21375-21383; Gum, et
7 al. (1994), *J. Biol. Chem.* 269:2440-2446), *MUC 5AC* (Meerzaman, et al. (1994), *J. Biol.*
8 *Chem.* 269:12932-12939), *MUC 5B* (Desseyn, et al. (1997) *J. Biol. Chem.* 272:3168-
9 3178) and *MUC 6* (Toribara, et al. (1997) *J. Biol. Chem.* 272:16398-16403) as well as in
10 rat (Ohmori, et al. (1994) *J. Biol. Chem.* 269:17833-17840), pig (Eckhardt, et al. (1991)
11 *The Journal of Biological Chemistry*, 266(15):9678-9686), cow (Bhargava, et al. (1990)
12 *Proc. Nat. Acad. Sci. U. S. A.* 97:6798-6802) and frog (Probst, et al. (1990) *Biochemistry*
13 29:6240-6244) mucins. The cysteine-rich domains in mucins show varying degrees of
14 similarity to the D-domains of von Willebrand factor (vWF).

15 Cystic fibrosis ("CF") commonly occurs among Caucasians (approximately 1 in
16 2,000 newborns). The mode of inheritance is autosomal recessive and about 5% of the
17 normal population carries the defective gene. Affected individuals can generally live
18 with reasonable lung function until the onset of a chronic bacterial infection. Almost all
19 patients contract either *Pseudomonas aeruginosa* or *Staphylococcus aureus* infections.
20 Mucus overproduction resulting from the bacterial infection damages lung function
21 directly by plugging airways and indirectly by shielding the bacteria from endogenous
22 and exogenous antibacterial agents. This creates a "wound that does not heal" and causes
23 chronic influx of inflammatory cells whose proteases degrade gas exchange tissue.
24 Respiratory function declines relentlessly until death results.

25 Current treatments fail to effect the complete eradication or prevention of these
26 bacterial infections in cystic fibrosis patients nor do they ameliorate the overproduction
27 of mucus. In addition, antimicrobial therapy using antibiotic therapeutic protocols have
28 complications. Patients with CF dispose of antimicrobial agents more rapidly than do
29 non-CF individuals, which results in the use of higher doses than those normally
30 recommended. Strains of *Pseudomonas aeruginosa* ("PA") can dissociate into multiple
31 serotypic forms, which often have different antimicrobial susceptibility patterns. Since

1 PA infection is chronic and the infecting strains of *Pseudomonas aeruginosa* are rarely
2 eradicated, resistance to multiple antimicrobial agents frequently develops, thwarting
3 antibiotic therapies. Moreover, therapeutic levels of antimicrobial agents in sputum are
4 difficult to achieve because of poor penetration and inactivation. Mucoïd
5 exopolysaccharides of mucoïd PA strains additionally present a barrier to penetration of
6 some antibiotics. Finally, allergy to certain antibiotics (such as betalactam) precludes
7 antibiotic therapy with some patients. Thus, as it is virtually impossible to eradicate the
8 bacteria, it is important to find alternate therapies to improve lung function and prolong
9 life. The ability to control mucin production may provide an alternative route to prevent
10 or alleviate airway plugging.

11 In addition to its role in exacerbating pulmonary infections in cystic fibrosis
12 patients, mucin overproduction is also a debilitating feature of chronic bronchitis,
13 bronchial pneumonia and chronic asthma. Smoking is the most important risk factor for
14 chronic bronchitis. Individuals dying *in status asthmaticus* are always observed to have
15 mucus-obstructed airways.

16 Consequently, there is a need to provide therapies for reducing mucus production
17 in individuals suffering from airway diseases such as cystic fibrosis, chronic bronchitis,
18 bronchial pneumonia and asthma.

19

20 Relevant Literature

21 Recent work has suggested that MUC 4 and MUC 5AC are the most highly
22 expressed mucins in the upper airway (Gendler, et al. (1996) *Pediatric Pulmonology*
23 13S:290 Abstract). Mucus secretions in the airway are produced from two different
24 secretory cell populations, surface epithelial goblet cells and the mucous cells in the
25 submucosal glands. MUC 5AC has been reported to be expressed primarily in the goblet
26 cells (Hovenberg, et al. (1996) *Biochem. J.* 318:319-24).

27 Increased expression of some members of the mucin family in response to certain
28 effectors has been reported. Recent reports have indicated that MUC 2, MUC 4 and
29 MUC 5B are expressed at a higher level in the airways of cystic fibrosis patients
30 compared with non-CF patients (Gendler et al. (1996) *Pediatric Pulmonology* 13S:290
31 Abstract); Li, et al. (1997) *Proc. Nat. Acad. Sci. (USA)* 94:967-972). Steiger et al. (1995)

1 (Am. J. Resp. Cell and Molec. Biol. 12:307-314) have reported that bacterial endotoxin
2 stimulates both the storage and release of a mucin-like molecule in airway epithelial cells.
3 Levine, et al. (1995) (Am. J. Resp. Dis. 12:196-204) have reported an increase in the
4 steady state levels of MUC 2 mRNA in NCI-H292 cells by exposure to tumor necrosis
5 factor- α . Li et al. (Proc. Natl Acad. Sci. (1997) 94:967-972) have reported that PA
6 lipopolysaccharide ("LPS") can increase transcription of the MUC 2 gene in epithelial
7 cells. Pre-incubation of the cells with the protein tyrosine kinase inhibitor Genistein
8 abolished the increase in transcription. Recent work in the laboratory of some of the
9 present inventors has suggested that MUC 5AC transcription is also increased after
10 exposure to various Gram-negative and Gram-positive bacteria in both bronchial explants
11 and cultured airway epithelial cells. Borchers and Leikauf have recently reported (Am. J.
12 Respir. Critical Care Med. 155:A778 (1997)) that exposure of rats to acrolein, a low
13 molecular weight aldehyde found in tobacco smoke, results in an increase in MUC-2
14 mRNA and concurrent secretion of mucin in airway tissue.

15 Partial MUC 5AC cDNA sequences have been reported (Meerzaman, et al. (1994)
16 J. Biol. Chem., 269:12932-12939); Guyonnet-Duperat, et al. (1995) Biochem. J. 211-
17 209).

18 The nucleotide sequence of human gastric mucin cDNA, HGM-1, from nucleotides 1942-
19 2281 is 99% identical to the MUC 5AC clone JUL 32 (Guyonnet-Duperat, et al. (1995)
20 Biochem. J. 211-209) and the sequence from nucleotides 2190-2541 is 92% identical to
21 the 5' end of MUC 5AC clone NP3a (Klomp, et al. (1995) Biochem. J., 308:831-80).

22 The sequence of the MUC 2 gene promoter has been reported (Velcich, et al. (1997) J.
23 Biol. Chem. 272:7968-7976 (1997).

24

25 SUMMARY OF THE INVENTION

26 The present invention provides methods and compositions for the selection of
27 inhibitors of mucin production. The present inventors have identified for the first time
28 the regulatory elements of the human MUC 5AC gene which control the response of the
29 gene to effectors which induce the expression of MUC 5AC. Nucleotide sequence of the
30 regulatory region is provided herein. By using these regulatory elements, particularly in
31 combination with a readily detectable reporter gene, it is possible to identify compounds

1 that inhibit the induction of mucin genes, particularly MUC 5AC, by various effectors, or
2 mucomones. Inhibitor compounds thus identified are useful in therapeutic methods for
3 the treatment of diseases and conditions associated with the inappropriate production of
4 mucin.

5 The present inventors have additionally discovered that tobacco smoke, in the
6 form of smoke-conditioned culture medium, can serve as a mucomone to induce the
7 transcription of mucin genes, in particular, the MUC 5AC gene. Nucleotide sequences
8 important for providing induction of MUC 5AC by tobacco smoke are identified.

9 The present invention thus provides polynucleotide molecules comprising one or
10 more MUC 5AC mucomone response elements, mucomone-inducible reporter gene
11 constructs comprising the MUC 5AC mucomone response elements operably linked to a
12 reporter gene, and cells comprising the reporter gene constructs.

13 The present invention additionally provides methods for identifying compounds
14 that inhibit mucin production using the polynucleotide molecules, reporter gene
15 constructs and cells of the present invention. In one embodiment, the method of the
16 present invention comprises contacting cells comprising a mucomone-inducible reporter
17 gene construct with a test compound, contacting said cells with a mucomone, and
18 determining the difference in reporter gene activity in response to mucomone in the
19 presence and absence of the test compound. The method is particularly useful for
20 identifying compounds that inhibit tobacco smoke-induced mucin production.

21 Mucomone-induced mucin production can be affected in a variety of ways, for
22 example, by inhibiting binding of mucomones to an epithelial cell or by inhibiting any
23 crucial step in the signal transduction cascade between binding of a mucomone and
24 mucin gene activation, including inhibiting transcription factors that interact with mucin
25 promoters. Such inhibitors can be used in treatments of medical conditions related to the
26 inappropriate expression of mucin, such as, for example, cystic fibrosis, chronic
27 bronchitis, bronchial pneumonia and bronchial asthma. The invention includes methods
28 and compositions related to drug discovery and therapeutic treatments.

29 Another embodiment of the invention provides for methods for treating an animal
30 by inhibiting the production of mucin in epithelial cells, particularly airway epithelial
31 cells, by administering an effective amount of an inhibitor of mucomone-induced mucin

1 production to the animal. Other methods, compounds and compositions are more fully
2 described herein.

3 4 5 BRIEF DESCRIPTION OF THE DRAWINGS

6 Figures 1A and 1B show the DNA sequence of the 5' regulatory region of the
7 human MUC 5AC gene (SEQ ID No: 1), from -3752bp to +83bp, +1bp being the
8 transcription start site. The transcription start site and the translation start sites (ATG) are
9 underlined .

10 Figure 2 is a bar graph showing the dose-dependent and time-dependent response
11 of M4-2 transfected HM3 cells to environmental tobacco smoke (ETS) - exposed
12 medium. The duration of exposure to the ETS medium is shown in hours on the
13 horizontal axis. Relative luciferase activity is shown on the vertical axis.

14 Figure 3 is a bar graph showing the dose-dependent and time-dependent response
15 of M4-2 transfected NCIH292 cells to environmental tobacco smoke (ETS) - exposed
16 medium. The duration of exposure to the ETS medium is shown in hours on the
17 horizontal axis. Relative luciferase activity is shown on the vertical axis.

18 Figure 4 is a bar graph showing effect of various inhibitors on the RLA induced
19 by exposure to the ETS - medium.

20 Figures 5A and 5B show the nucleotide sequence of the MUC 5AC cDNA (SEQ
21 ID No. 2).

22 Figure 6 shows the nucleotide sequence of a MUC 5AC genomic clone from -
23 16bp to +412bp (SEQ ID No:22). The transcription start site is designated +1bp and is
24 underlined. The first exon is shown in bold. The putative translation start ATG is
25 underlined. An intron-exon junction occurs between +136 and +137.

26 27 DESCRIPTION OF SPECIFIC EMBODIMENTS

28 **DEFINITIONS**

29 Mucin or mucins typically refer to the structural protein components of mucus
30 from epithelial cells that protect tissues, such as the respiratory and reproductive tracts.
31 Typically, mucins form extremely large oligomers through linkage of glycoprotein

1 monomers using disulfide bonds. Usually, such glycoproteins are large >100,000 daltons
2 and typically consist of approximately 75% carbohydrate and 25% protein. Mucins
3 include proteins encoded by the MUC genes described herein. Altered mucins, which
4 contain abnormal concentration of sulfate, sialic acid or fucose, also occur in pathological
5 conditions, such as inflammatory diseases.

6 Mucomones refer to molecules that induce or effect, directly or indirectly, mucin
7 production and typically include proteins, amino acids, simple sugars, complex sugars,
8 lipopolysaccharides ("LPS") and other pathogen exoproducts, irritants like tobacco
9 smoke and constituents of smoke such as acrolein.

10 By mucomone-induced mucin production is meant an increase in mucin
11 production in a cell in response to the presence of a mucomone. In some instances, a
12 reporter gene will be substituted for a mucin gene and in these cases mucomone-induced
13 mucin production can be determined with reference to the increase in reporter gene
14 activity in response to the presence of a mucomone.

15 By reporter gene activity is meant any activity associated with expression of the
16 reporter gene including the transcription or translation of the gene or the presence or
17 activity of the gene product.

18 Production, when used in the context of describing a cellular process, typically
19 refers to a cellular process or processes involved in maintaining the steady state level of a
20 molecule, such as a mucin, for example MUC 5AC. Consequently, production includes
21 the cellular processes of gene activation or induction, transcription, protein synthesis, and
22 in appropriate instances, protein modification and/or secretion. Production also refers to
23 cellular and extracellular processes responsible for maintaining steady state levels of a
24 molecule, such degradation pathways and extracellular structural elements that anchor
25 molecules to cells or in an extracellular matrix.

26 Secretion, when used in the context of describing a cellular process, typically
27 refers to a cellular process or processes of transporting a molecule from inside the cell to
28 an extracellular location.

29 By operably linked, as used herein in the context of assembly of the reporter gene
30 construct, is meant that the component sequences are joined in such fashion that they
31 function together to achieve the intended purpose. For example, a mucomone response

1 element is operably linked to a responsive promoter when the promoter is activated in
2 response to the effector (mucomone) to which the mucomone response element responds.
3 A reporter gene is operably linked to a promoter when transcription of the reporter gene
4 from the promoter can occur.

5 Protein synthesis, when used in the context of describing a cellular process,
6 typically refers to a cellular process or processes involved in making a molecule, such as
7 a mucin, for example MUC 5AC. Protein synthesis may involve transport of the
8 molecule within the cell making the molecule. The term protein synthesis, however, does
9 not include reference to the term secretion described herein.

10 Transcriptional control element refers to a nucleotide sequence which is involved
11 in the regulation of transcription of a gene or genes. Transcriptional control elements
12 include mucomone response elements, promoters, enhancers, and the like.

13 Mucomone response element refers to a nucleotide sequence which, when
14 operably linked to a responsive promoter, results in the activation of transcription from
15 the promoter in response to a mucomone. A responsive promoter is one that is capable of
16 activation by a mucomone response element. Activation of transcription includes either
17 initiation of transcription or increase in the rate or amount of transcription.

18 Treatment of a disease or condition refers to procedures for the amelioration or
19 eradication of a disease or condition, or of symptoms associated with a disease or
20 condition, and includes the prevention of the development of such disease or condition or
21 of symptoms associated with such disease or condition.

22

23 **Description of Embodiments**

24 Overproduction of mucins has been implicated as a factor in a number of diseases
25 or conditions of the airway including chronic bronchitis, bronchial pneumonia, asthma
26 and bacterial infections associated with cystic fibrosis. Exposure of airway cells to
27 various external stimulants, such as *Pseudomonas aeruginosa* lipopolysaccharides, has
28 been shown to result in increased mucin production. Recent reports suggest that MUC
29 5AC is one of the most highly expressed mucins in the upper airway and is expressed
30 primarily in goblet cells.

31 The present inventors have for the first time isolated and sequenced the 5'

1 regulatory region of the human MUC 5AC gene. Mucomone response elements have
2 been identified within the 5' regulatory region which are responsible for the increase in
3 transcription of the MUC 5AC gene, and consequent increase in mucin production, *in*
4 *vivo*, in response to certain effectors, herein called mucomones. The MUC 5AC
5 mucomone response elements, in combination with a responsive promoter, can effect an
6 increase in activity of an associated gene in response to the presence of a mucomone. A
7 mucomone-inducible reporter gene construct can be prepared in which reporter gene
8 activity is induced by the presence of mucomone. Such constructs are useful in a method
9 for the identification of compounds that inhibit mucomone-induced mucin production.
10 Inhibitor compounds that are identified are useful in a method for inhibiting mucin
11 production in animals.

12 The 5' regulatory region of the human MUC 5AC gene, including one or more
13 mucomone response elements, is present on an approximately 4 kilobase pair ("4KB")
14 DNA fragment which is immediately adjacent to, and upstream from, the MUC 5AC
15 transcription initiation site. This 4KB fragment has now been identified, isolated and
16 sequenced. A vector containing the cloned 4KB fragment was deposited with the
17 American Type Culture Collection, 10801 University Blvd., Manassas, VA 20110 as
18 ATCC 98701 on March 26, 1998. Nucleotide sequence of 3753 nucleotides from the
19 region of the 4KB fragment immediately adjacent to the transcription initiation site is
20 provided in Figure 1A and B (SEQ ID No. 1). The 4KB fragment contains one or more
21 mucomone response elements that are responsible for the increase in MUC 5AC
22 transcriptional activity in the presence of a mucomone, particularly environmental
23 tobacco smoke or *P. aeruginosa*-conditioned medium. The 4KB MUC 5AC 5'
24 regulatory region fragment is readily identified and isolated using the information
25 disclosed herein by methods that are well-known in the art. For example, the 4KB
26 fragment may be identified by hybridization of a human genomic library with a DNA
27 fragment probe comprising at least 20 consecutive nucleotides, preferably 50 consecutive
28 nucleotides, more preferably 100 consecutive nucleotides of the sequence of Figure 1A
29 and B (SEQ ID No. 1), or the complement of that sequence. The DNA fragment probe is
30 conveniently prepared by methods that are well known in the art, for example by
31 chemical synthesis. Preparation of a human genome library and screening for clones

1 containing the 4KB MUC 5AC 5' regulatory region fragment by hybridization can be
2 carried out by well known methods, for example, as described in Sambrook et al.
3 (Molecular Cloning, Cold Spring Harbor Laboratories 1989). Alternatively, a DNA
4 fragment comprising the sequence of the 4KB MUC 5AC 5' regulatory region, or
5 fragments thereof, may be synthesized using the polymerase chain reaction using primers
6 based on the sequence disclosed herein for this region and the complement of that
7 sequence. Methods for designing primers for amplification of particular sequences, as
8 well as the PCR protocols, are well known in the art, see for example, PCR Protocols: A
9 Guide to Methods and Applications, Innis et al., eds., Academic Press, 1990. The entire
10 4KB fragment, or a portion thereof comprising at least one mucomone response element,
11 is operably linked to a reporter gene by conventional molecular biological methods to
12 provide the mucomone-inducible reporter gene constructs of the present invention.

13 The present invention includes an isolated polynucleotide molecule comprising at
14 least 20 consecutive nucleotides, preferably at least 50 consecutive nucleotides, most
15 preferably at least 100 or more consecutive nucleotides, of the 4KB MUC 5AC 5'
16 regulatory region (SEQ ID No:1) or a complement of that sequence. The nucleotide
17 sequence of 3753 bases of the MUC 5AC 5' regulatory region immediately upstream of
18 the MUC 5AC transcription initiation site is shown in Figure 1A and B(SEQ ID No. 1).
19 The MUC 5AC transcription initiation site is underlined and is designated as nucleotide
20 +1. The 5' regulatory region is numbered counting backward from the transcription
21 initiation site (that is, from right to left) from -1bp to -3752bp. A TATA box is present at
22 -28 through -31. Thus, the 4KB MUC 5AC 5' regulatory region fragment shown in
23 Figure 1A and B contains the MUC 5AC promoter. Figure 5 shows 3358 nucleotides of
24 the sequence of a MUC 5AC cDNA (SEQ ID No. 2) beginning with the transcription
25 initiation site. The transcription initiation site is again designated as nucleotide +1. The
26 cDNA sequence is numbered counting forward (that is, left to right) from +1 to +3358.
27 The ATG translational start codon is underlined. Figure 6 shows the genomic sequence
28 of MUC 5AC from just before the transcription initiation site (-16bp) through the first
29 exon into the first intron (+412).

30 The polynucleotide molecule of the present invention is useful as a DNA
31 fragment probe for identification and isolation of the 4KB MUC 5AC 5' regulatory

1 region. The polynucleotide molecule of the present invention may also be operably
2 linked to a reporter gene to provide a mucomone-inducible reporter gene construct. The
3 polynucleotide molecule of the present invention may include the entire 4KB MUC 5AC
4 5' regulatory region fragment, or portions thereof comprising at least 20 consecutive
5 nucleotides, preferably 50 consecutive nucleotides, more preferably 100 or more
6 consecutive nucleotides of the nucleotide sequence of Figure 1A and B (SEQ ID No. 1).
7 For use in combination with a reporter gene, the polynucleotide molecule chosen will
8 preferably contain one or more mucomone response elements. The polynucleotide
9 molecule can be prepared in any of a number of ways that are well known in the art, for
10 example by chemical or enzymatic synthesis (for example, PCR). The polynucleotide
11 molecule may be prepared by fragmentation of larger DNA fragments (for example, the
12 4KB MUC 5AC 5' regulatory region fragment), for example, by restriction digestion or
13 digestion with other endonucleases or exonucleases or by mechanical shearing.

14 From the disclosure of the sequence of the 5' regulatory region of the MUC 5AC
15 gene now provided, a mucomone response element can be identified by methods that are
16 well-known in the art or by methods described herein. In general, a first reporter gene
17 construct can be made comprising a sequence of at least 20 consecutive nucleotides of the
18 4KB MUC 5AC 5' regulatory region fragment operably linked to a responsive promoter,
19 for example the MUC 5AC promoter as identified herein, and the coding region of a
20 reporter gene. The first reporter gene construct will preferably comprise DNA fragments
21 having the sequence of at least 20 consecutive nucleotides, preferably 50 consecutive
22 nucleotides, more preferably 100-200 consecutive nucleotides, most preferably 500 or
23 more consecutive nucleotides, of the 4KB MUC 5AC 5' regulatory region (SEQ ID
24 No:1). Reporter gene activity in response to the presence of a mucomone can be
25 determined by any of a variety of methods as described herein. For comparison, the
26 response of a control reporter gene construct is determined. The control reporter gene
27 construct is similar to the first reporter gene construct but does not contain sequences
28 from the 4KB MUC 5AC 5' regulatory region but may contain the MUC 5AC promoter.
29 When the relative reporter gene activity in response to mucomone of the first reporter
30 gene construct is at least two-fold greater than that of the control reporter gene construct,
31 a mucomone response element is present in the first reporter gene construct.

1 A mucomone response element may respond to a variety of different mucomones
2 or may be specific for particular mucomone or a limited group of mucomones. A
3 mucomone response element may respond differently to different mucomones.
4 Preferably, in identifying a mucomone response element for use in the method of the
5 present invention to identify inhibitors of mucomone-induced mucin production, the
6 response element will be selected for response to the particular mucomone or mucomones
7 to be employed in the method to identify inhibitor compounds.

8 A mucomone response element can be operably linked to a responsive promoter
9 and a reporter gene to provide the mucomone-inducible reporter gene construct of the
10 present invention. By mucomone-inducible is intended an increase in reporter gene
11 activity in response to the presence of a mucomone. It will be apparent that the increase
12 in reporter gene activity in response to a mucomone can be observed only if the construct
13 is present in a cell which is capable of mucomone-induced mucin production.

14 A cell is capable of mucomone-induced mucin production if it contains the
15 components necessary to provide increased transcriptional activity of a mucin gene or
16 genes in response to the presence of a mucomone. Typically, cells of the type which
17 produce mucin *in vivo*, particularly those cells which produce MUC 5AC protein *in vivo*,
18 will be capable of mucomone induced mucin production. In particular, certain epithelial
19 cells or cell lines are capable of mucomone-induced mucin production and are useful in
20 the practice of the present invention.

21 The mucomone-inducible reporter gene construct of the present invention is
22 useful in a method for identifying an inhibitor of mucomone-induced mucin production.
23 In one embodiment, the method of the present invention comprises the steps of (a)
24 contacting cells comprising a mucomone-inducible reporter gene construct with a
25 mucomone, wherein said reporter gene construct comprises a mucomone response
26 element comprising at least 20 consecutive nucleotides of the 4KB MUC 5AC 5'
27 regulatory region (SEQ ID No:1) and a responsive promoter, operably linked to a reporter
28 gene, (b) contacting said cells with a test compound, and comparing the reporter gene
29 activity after performing steps (a) and (b) with the reporter gene activity after performing
30 only step (a).

31 The present invention recognizes that epithelial cells involved in mucin

1 production would be useful components of *in vitro* methods for identifying compounds
2 that are useful as therapeutics, such as compounds that inhibit mucomone-induced mucin
3 production. Although not essential for practicing the invention, such identifying methods
4 can involve screening assay systems that permit high throughput automated screening.

5 Such methods include the use of mucomones that can induce mucin production in
6 the cell type employed in the method. Examples of such mucomones include, but are not
7 limited to, bacterial conditioned medium from both Gram-negative and Gram-positive
8 bacteria, including PA conditioned media, *E. coli* conditioned media, LPS from PA, *E.*
9 *coli* and other Gram-negative bacteria, Lipid A from PA, *E. coli* and other Gram-negative
10 bacteria, irritants such as tobacco smoke and constituents of smoke such as acrolein, as
11 well as smoke-exposed culture medium, and other mucomones known in the art or
12 described herein. The preparation of bacterial conditioned medium is well known in the
13 art (See, for example, Li et al. 1997, *supra*) and can be readily made by growing a
14 bacterial culture in an appropriate culture medium, removing the bacteria after a suitable
15 time by, for instance centrifugation, and removing any residual viable bacteria from the
16 culture medium by, for example, ultrafiltration. Mucomones that are particularly useful
17 in the practice of the present invention include conditioned growth medium from any
18 Gram-negative or Gram-positive bacteria, or specific components thereof such as LPS;
19 environmental tobacco smoke-conditioned medium, or specific components thereof; IL1,
20 TNF- α , other inflammatory mediators, forskolin, TPA, dibutyl cAMP, ceramide.
21 Preferred mucomones include bacterial LPS, smoke-conditioned medium and bacterial
22 conditioned medium.

23 Typically, in the method of the present invention, mucomone will be added to
24 cells after exposure of the cells to a test compound but may be added simultaneously with
25 the test compound or may be added prior to the addition of test compound. It is within
26 the competence of one of ordinary skill in the art to determine the preferred order of
27 addition for any particular combination of mucomone and test compound. Likewise, the
28 manner of addition and the amount of mucomone added will vary with the particular
29 mucomone and are readily determinable by one of ordinary skill in the art.

30 The method of the present invention can be used to screen any of a number of
31 compounds for the ability to inhibit mucomone-induced mucin production, including, but

1 not limited to, tyrosine kinase inhibitors, protein kinase A inhibitors, mitogen-activated
2 protein (MAP) kinase inhibitors, inhibitors of mucomone binding, and other types of
3 compounds.

4 A number of methods may be used to detect mucin production or mucin gene
5 activation, indirectly or directly. For instance, mucin production can be directly detected
6 by measuring mucin protein synthesis or by measuring mucin in cell supernatants or
7 airway lavage fluid, and mucin gene activation can be directly detected by measuring
8 transcription or RNA levels using the appropriate labels or probes for such assays. For
9 example, an RNase protection assay (RPA), as described herein and known in the art, can
10 be used to study the effect of a compound on the transcriptional activity of a MUC gene
11 by detecting changes in the RNA levels of one or more of the known mucin genes
12 (Gendler, et al. (1990) *J. Biol. Chem.* 265:15286-15293; Gum, et al. (1994) *J. Biol.*
13 *Chem.* 269:2440-2446; Velcich, et al. (1997) *J. Biol. Chem.* 272:7968-7976); Gum, et al.
14 (1990) *Biochemical and Biophysical Research Communications* 171:407-415; Aubert et,
15 al. (1991) *American Journal of Respiratory Cell and Molecular Biology* 5:175-185; Rose,
16 et al. (1989) *J. Biol. Chem* 264:8193-99; Toribara, et al. (1993) *J. Biol. Chem.* 268:5879-
17 5885; Reddy, M.S. (1992) *Biochemical J.* 287:639-43; Sachdev (1994) *Biochemical J.*
18 300 (pt 2):295-298). Mucin probes can be selected from among the known nucleotide
19 sequences for mucin, such as for human mucins, and can be used for RPA or traditional
20 hybridization techniques, such as, for example, Northern blots. Preferably, probes
21 specific for MUC 5AC are used. Oligonucleotides comprising known mucin nucleotide
22 sequences can also be used to measure RNA levels by quantitative PCR. The methods
23 and sequences of the above-referenced publications are herein incorporated by reference.
24 Direct detection methods may also include antibody assays for mucins to assess
25 regulation at the protein level, such as the use of A10G5 monoclonal antibody to detect
26 MUC-2 (Finkbeiner, et al. (1988) *Am. J. Pathol.* 131:290-297). Monoclonal or
27 polyclonal antibodies to MUC 5AC may be prepared by methods that are well known in
28 the art. It should be remembered that mucin secretion is not a desirable method for
29 detecting mucin production because mucin secretion from the cells, especially mucin
30 secretion within 2-3 hours in response to a mucomone, is not indicative of gene
31 upregulation. Such rapid secretion measurements provide a better measurement of release

1 of stored mucin from the cell and not synthesis of a large molecule comprised both of
2 protein and sugar, which often has a total molecular weight of 1,000,000 daltons.

3 Conveniently, a reporter gene assay can be used to indirectly determine the
4 amount of mucomone-induced mucin production. An increase in reporter gene activity in
5 response to mucomone is indicative of mucomone-induced mucin production. As will be
6 readily apparent, such an assay can also be used to determine the inhibition of
7 mucomone-induced mucin production by various inhibitors. The assay is typically
8 carried out using an epithelial cell comprising a mucomone-inducible reporter gene
9 construct as described herein. Reporter gene activity is determined by methods
10 appropriate for the particular reporter gene chosen. For example, where the reporter gene
11 construct contains a luciferase gene or a chloramphenicol acetyltransferase gene as the
12 reporter gene, luciferase or chloramphenicol acetyltransferase (CAT) assays,
13 respectively, can be used to determine the amount of mucomone-induced reporter gene
14 activity and the inhibition of that mucomone-induced activity in the presence of a test
15 compound.

16 A reporter gene includes any gene that expresses a detectable gene product, which
17 may be RNA or protein. Preferred reporter genes are those whose gene products are
18 readily detectable. The reporter gene may also be included in the construct in the form of
19 a fusion with a gene that includes desired transcriptional regulatory sequences or exhibits
20 other desirable properties. For example, reporter gene fusion with a mucin gene may be
21 used. Many reporter genes and transcriptional regulatory elements are known to those of
22 skill in the art and others may be identified or synthesized by methods known to those of
23 skill in the art.

24 Examples of useful reporter genes include, but are not limited to CAT
25 (chloramphenicol acetyl transferase) (Alton and Vapnek (1979) *Nature* 282:864-869),
26 luciferase, and other readily detectable enzyme systems, such as beta-galactosidase,
27 firefly luciferase (deWet, et al. (1987) *Mol. Cell. Biol.* 7:725-737); bacterial luciferase
28 (Engebrecht and Silverman (1984), *PNAS* 1:4154-4158; Baldwin, et al. (1984)
29 *Biochemistry* 23:3663-3667), alkaline phosphatase (Toh, et al. (1989) *Eur. J. Biochem.*
30 182:231-238, Hall et al. (1983) *J. Mol. Appl. Gen.* 2:101), and GFP (green fluorescent
31 protein) (Chalfie, et al. (1994) *Science* 263:802-805).

1 The mucomone-inducible reporter gene construct is assembled by methods that
2 are well known in the art. The assembly is most conveniently accomplished by ligation
3 of DNA fragments, for instance restriction fragments or chemically or enzymatically
4 synthesized DNA fragments. Alternatively, the various DNA sequences may be
5 chemically or enzymatically synthesized as a single fragment. Expression of the reporter
6 gene can be monitored in any of a number of ways that are well known in the art
7 including, for example, by measuring transcription or translation of the reporter gene or
8 the presence or activity of the reporter gene product.

9 Mucomone-inducible reporter gene constructs can be prepared by operably
10 linking a reporter gene or reporter gene coding region with a portion of the 4KB 5'
11 regulatory region fragment of the MUC 5AC gene containing one or more mucomone
12 response elements and a responsive promoter. Typically, the portion of the 4KB 5'
13 regulatory region chosen will also contain the MUC 5AC promoter. Alternatively, the
14 MUC 5AC promoter may be joined to a portion of the 4KB MUC 5AC regulatory region
15 that is not adjacent to the MUC 5AC promoter in the native sequence. For example, a
16 portion of the 4KB MUC 5AC regulatory region from -3752bp to -3555bp of the
17 sequence of Figure 1A and B may be joined directly to a fragment containing the
18 promoter, for instance, the portion from -50bp to +1bp. Other promoters, for example, a
19 MUC 2 promoter or a thymidine kinase promoter, may be used in place of, or in addition,
20 to the MUC 5AC promoter, provided that the particular promoter chosen is responsive to
21 the mucomone response elements. Preferably, the reporter gene construct includes the
22 MUC 5AC promoter and at least one mucomone response element from the 4KB MUC
23 5AC 5' regulatory region. For example, the reporter gene construct will include a
24 mucomone response element contained within the polynucleotide molecule having the
25 nucleotide sequence of Figure 1A and B (SEQ ID No:1) and identified by the procedure
26 already described. Any portion of the 4KB MUC 5AC 5' regulatory region, up to the
27 entire 4KB fragment (that is, from -3752bp to -1bp of the sequence of Figure 1A and B,
28 which corresponds to nucleotides 1-3753 of SEQ ID No:1), that contains one or more
29 mucomone response elements may be used in making the reporter gene construct. The
30 polynucleotide molecules containing the mucomone response elements can be operably
31 linked to the MUC 5AC promoter or to another responsive promoter, such as, for

1 example, the thymidine kinase (tk) promoter, to render such heterologous promoters
2 responsive to mucomone stimulation. The reporter gene constructs will demonstrate at
3 least a 2-fold increase in reporter gene activity, preferably a 5-fold increase or greater,
4 more preferably a 10-fold increase or greater and most preferably a 50-fold increase or
5 greater in response to mucomone.

6 The mucomone-inducible reporter gene construct may contain additional
7 transcriptional regulatory elements or other sequences, that are not necessarily regulated
8 by the mucomone or the mucomone receptor, but are selected for their ability to reduce
9 background level transcription or to amplify the transduced signal and to thereby increase
10 the sensitivity and reliability of the assay. In addition, sequences useful for transfection
11 and selection of transfectants may be included. For example, selectable marker genes
12 such as a gene for neomycin resistance or for G418 resistance, or sequences that provide
13 homology with a host target sequence for homologous recombination of the vector into
14 the host sequence can be present in the reporter gene construct.

15 Any cell, particularly an epithelial cell, that produces mucin in response to a
16 mucomone can be used as a component of the assays described herein. Although most
17 epithelial cells will exhibit mucomone inducible mucin production, the regulation of
18 mucin production can vary from tissue to tissue as well as depend on the type of
19 mucomone. Thus, if an inhibitor of colon or airway mucin production is desired, it will
20 often be preferable to use colon or airway derived mucin producing cells in identifying
21 such an inhibitor. If it is not known whether a cell produces mucin in response to a
22 mucomone, mucomone-induced mucin production can be easily determined using the
23 assay described herein. In brief, the cell is contacted with a mucomone and mucin
24 production is measured before and after exposure of the cell to mucomone. Mucin
25 production can be measured by any of the methods described herein and any other
26 methods known in the art. Cells that are identified as producing mucin in response to
27 mucomone in such assays can then be used in a method for identifying therapeutic
28 compounds. Choice of cells is not restricted to this manner of selection and it will often
29 be the case that some cell lines will be preferred irrespective of the tissue source, such as
30 HM3 and NCIH292. Preferred cells for the practice of the present invention are those
31 that respond well to mucomones, are convenient to culture, and produce large reporter

1 gene signals in the presence of a mucomone compared to in the absence of a mucomone,
2 i.e. cells that have a low level of mucin production in the absence of a mucomone.
3 Preferably, NCIH292, HM3, Hela, CFTE290, NCIH292 and 16Lu cells are used in the
4 identification methods and most preferably HM3 and NCIH292 are used. Cells from a
5 variety of epithelial tissues can be used as well, such as airway secretory cells, ciliated
6 cells, epithelial cells of the respiratory tract, kidney secretory cells and reproductive tract
7 epithelial cells. Once a particular cell type has been selected, various direct and indirect
8 detection assays of mucin production can be used, as described herein.

9 For use in the method of the present invention, the cells are transfected with a
10 reporter gene construct by any of a number of methods that are well known in the art, for
11 example as described in Felgner, et al. *Proc. Natl Acad. Sci.* 84:7413 (1987), Wigler, et
12 al. (1977) *Cell* 11:233, Neumann, et al. (1982) *EMBO J.* 1:841-845, Sussman, et al.
13 (1984) *Mol. Cell. Biol.* 4:1641. The reporter gene construct may be transiently present in
14 the cell or may be stably incorporated into the chromosome of the cell. Preferably, the
15 reporter gene construct is stably incorporated into the chromosome.

16 In the method of the present invention, a test compound can be assayed for its
17 ability to inhibit mucomone-induced mucin production in cells comprising a mucomone-
18 inducible reporter gene construct by measuring a change in reporter gene activity in the
19 presence and absence (control) of the compound being tested. It will be recognized that
20 such controls can be used in any of the assays described herein and that other controls can
21 be readily interchanged to achieve specific detection, such as using cells without
22 functional 5' regulatory regions of the mucin gene operably linked to a functional
23 reporter gene, by not adding a mucomone or by blocking the action of an added inhibitor.
24 The method is typically carried out using an epithelial cell line in which a mucomone-
25 inducible reporter gene construct is stably incorporated into the chromosome. The
26 method may alternatively be carried out using cells that are transiently transfected with
27 the reporter gene construct. The cells comprising the reporter gene construct are
28 contacted with mucomone to activate the mucomone-induced reporter gene construct.
29 The cells are additionally contacted with the compound to be tested. The cells may be
30 exposed to the mucomone prior to addition of the test compound, simultaneously with the
31 addition of the test compound or at some time after the addition of the test compound.

1 Reporter gene activity in response to mucomone is measured after exposure to the test
2 compound and compared with reporter gene activity in the absence of the test compound
3 by methods described herein. It will be apparent that mucomone-induced reporter gene
4 activity may be optimized by varying assay parameters, such as concentration of
5 mucomone used, timing of addition of mucomone, etc. Optimization of mucomone-
6 induced reporter gene activity is well within the competence of one of ordinary skill in
7 the art and is best accomplished in separate assays prior to addition of test compounds. It
8 will also be apparent that the test compound may be used in a range of concentrations to
9 optimize the effect on mucomone-induced reporter gene activity.

10 The method of the present invention is typically carried out as follows. Cells
11 capable of mucomone-induced mucin production are transfected with a mucomone-
12 inducible reporter gene construct by standard procedures (for example, see Hiro et al. *J.*
13 *Cell. Biochem.* (1996) 61:350-362). The cells are incubated under standard conditions
14 following transfection. Approximately 40 hours after transfection, the test compound is
15 added to the cells. After exposure of the transfected cells to the test compound for about
16 two hours, a mucomone is added and incubation is continued for approximately an
17 additional 6 to 24 hours. The cells are then harvested and the activity of the reporter gene
18 is determined for the test sample. A control sample is prepared identically except that no
19 test compound is added at 40 hours. The amount of reporter gene activity in the test
20 sample is compared with that of the control sample. The reporter gene activity may be
21 normalized to some internal control, for example, total protein or the amount or activity
22 of a constitutive cellular protein. Alternatively, the reporter gene construct may contain a
23 second readily detectable gene, the activity of which can be used to normalize the
24 reporter gene activity. When the amount of reporter gene activity in the control sample is
25 greater than the amount of reporter gene activity in the test sample, an inhibitor of
26 mucomone-induced mucin production has been identified. Preferably, the control
27 reporter gene activity is at least two-fold greater than the test sample reporter gene
28 activity.

29 In addition to the transfected cells of the present invention, transgenic animals
30 comprising the mucomone-inducible reporter gene construct can be prepared. Methods
31 of preparing transgenic animals are well known and include, for example, microinjection

1 of a DNA molecule into the male pronucleus of a fertilized egg (Brinster et al. (1981)
2 *Cell* 27:223; Costantini, et al. (1981) *Nature* 293:540), introduction of recombinant viral
3 or retroviral molecules into an animal at a multi-cell stage, injection of a DNA molecule
4 into an embryonic stem cell and transplantation into a blastocyst (WO 91/19796).
5 Transgenic animals comprising the mucomone-inducible reporter gene construct can be
6 used to identify inhibitors of mucomone-induced mucin production *in vivo* by evaluation
7 of the reporter gene activation in response to mucomone in the presence and absence of
8 the inhibitor compound. Preferred reporter genes for use in the transgenic animal of the
9 present invention include β -galactosidase, human growth hormone and green fluorescent
10 protein.

11 Any of a number of compound types can be tested for inhibition of mucomone-
12 induced mucin production, including, but not limited to, inhibitors of mucomone binding,
13 tyrosine kinase inhibitors, protein kinase A inhibitors, MAP kinase inhibitors, inhibitors
14 of other components of the signal transduction pathway, inhibitors of transcription factors
15 that interact with mucin promoters, and other compound types. Inhibitors of mucomone
16 binding can include mucomone receptor antagonists such as, for example, LPS
17 antagonists. Inhibitors may include small molecule organic compounds, simple or
18 complex sugars, peptides, dominant negative mutants of proteins involved in mucomone
19 binding, signal transduction, and transcription, and other compounds. LPS antagonists
20 can include Lipid A analogs, lipid X analogs and other diglucosamine analogs, and other
21 compound types. Inhibitors of transcription factors include, for example, inhibitors of
22 NF- κ B, AP1, AP2, and C/EBP. Signal transduction inhibitors include inhibitors of src,
23 ras, raf1, mek1/2, erk1/2, and rsk. Inhibitors of src that are effective inhibitors of
24 mucomone-induced mucin production include pyrazolopyrimidine (PP1) and a dominant
25 negative mutant of src. Inhibitors of ras that are effective inhibitors of mucomone-
26 induced mucin production include a dominant negative mutant of ras, Ras N17 (Feig et
27 al. (1988) *Mol. Cell. Biol.* 8:2472). Inhibitors of mek1/2 that are effective inhibitors of
28 mucomone-induced mucin production include a PD98059 (Aless et al. (1993) *J. Biol.*
29 *Chem.* 270:27489) and a dominant negative mutant of mek1/2, HMEK K97R (Minden et
30 al. (1995) *Cell* 81:1147). Inhibitors of erk1/2 that are effective inhibitors of mucomone
31 production include tyrphostin (Novogrodsky et al. *Science* 264:1319) AG126. Inhibitors

1 of rsk that are effective inhibitors of mucomone production include a dominant negative
2 mutant of rsk (Ghoda et al. (1997) *J. Biol. Chem.* 272:21281).

3 Once it is determined that a compound inhibits mucomone induction of mucin
4 production, useful inhibitors can be selected. Selection criteria are usually based on the
5 extent of modulation produced by the tested compound. Compounds will usually be
6 selected on their ability to inhibit or reduce undesirable mucin production. Such
7 compounds will be useful for the treatment of medical conditions caused by inappropriate
8 mucin production or secretion. Such compounds will also be useful in treating, for
9 example, CF, chronic bronchitis, bronchial pneumonia or bronchial asthma. Typically,
10 compounds that inhibit mucomone-induced mucin production by at least 10%, preferably
11 by at least 30% and more preferably by at least 70% compared to control mucomone-
12 induced mucin production (that is, mucin production in the absence of test compound)
13 will be selected as useful compounds. Such percent inhibition criteria can be applied to
14 other measurements in assays described herein, such as detection of reporter gene activity
15 and RPA assays.

16 More specific selection criteria can be advantageously used to identify
17 compounds that more specifically modulate a cellular process. It will be recognized that
18 the affinity of the compound being tested for its receptor can often dictate the specificity
19 of the compound, such as a compound with an affinity for a cellular tyrosine kinase,
20 protein kinase A, MAP kinase, LPS, LPS binding protein, or an LPS receptor.
21 Consequently, it is desirable to select compounds that bind to receptors or modulate
22 function with a high apparent or actual affinity. In the case of tyrosine kinase inhibitors,
23 affinities of 10^{-3} or less are typical under physiological conditions, 10^{-5} or less are
24 preferred, and 10^{-6} are more preferred. To achieve such desired results, new compounds
25 or known tyrosine kinase inhibitors or LPS antagonists can be synthesized and screened
26 at predetermined concentrations. Typically, compound concentrations will be 500
27 micromolar or less, preferably 50 micromolar or less, more preferably 10 micromolar or
28 less, even more preferably 1 micromolar or less and most preferably 0.1 micromolar or
29 less. The percentage inhibition criteria discussed herein can be applied to these
30 concentration selection criteria.

31 Many different types of tyrosine kinase inhibitors (TKIs) can be tested for

1 inhibitory activity of mucomone-induced mucin production. Such TKIs are described
2 herein and known in the art. Tyrosine kinases are typically classified as either receptor
3 tyrosine kinases or non-receptor tyrosine kinases. Some TKIs inhibit both types of
4 tyrosine kinases and some TKIs, such as, for example, pyrazolopyrimidine (PP1) are
5 specific for non-receptor tyrosine kinases. Often, the TKI will either bind to the ATP or
6 tyrosine site on the tyrosine kinase. Many chemical structures that mimic ATP or
7 tyrosine binding to the tyrosine kinase can be used. For molecules that bind to the ATP
8 site (ATP analogs), it is desirable to test compounds that comprise at least an adenosine
9 base or adenosine base derivative. For molecules that bind to the tyrosine site (tyrosine
10 analog), it is desirable to test compounds that comprise at least a substituted benzene ring,
11 such as (HO)_n-benzene-X, where X can be a stable moiety of 1 to 30 atoms, usually
12 comprised of N, S, C, O, or H atoms. TKIs that are identified as inhibitors of mucomone-
13 induced mucin production can be used for the other methods described herein.

14 Although not necessary for the practice of the present invention, a test compound
15 identified in the reporter gene assay can be assessed for inhibitory activity in *in vivo*
16 assays. *In vivo* assays and measurements can be used to further select for useful
17 compounds. Alternatively, an *in vivo* assay can be used to identify compounds alone or
18 prior to an *in vitro* assay. For example, a test compound can be assayed *in vivo* by
19 examining its ability to inhibit mucin production in the airway of an animal upon
20 exposure to a mucomone. Alternatively, transgenic animals comprising the mucomone-
21 inducible reporter gene construct can be used in an *in vivo* assay. Such transgenic
22 animals will be exposed to mucomone in any appropriate fashion in the presence and
23 absence of a test compound and reporter gene activity evaluated by the methods
24 described herein.

25 The present invention recognizes that mucomones can directly induce mucin
26 production by epithelial cells, such as airway cells. Consequently, the methods of the
27 invention are directed to inhibiting inappropriate mucin production using compounds,
28 such as, for example, small molecule organic compounds, simple or complex sugars,
29 peptides, other inhibitors of signal transduction components, transcription factors, and
30 mucomone binding, and other compounds as described herein and are known in the art
31 that have been identified as inhibitors of mucin production. Such cells include airway

1 secretory cells, reproductive tract epithelial cells, ciliated cells, epithelial cells of the
2 respiratory tract and reproductive tract epithelial cells. Delivery of such inhibitors will
3 depend on the area to be treated. For example, treatment of the respiratory tract can be
4 achieved via aerosol or oral application. Intravenous application is a useful mode of
5 delivery and is sometimes preferred in emergency cases.

6 The invention includes a method of inhibiting mucin production in an animal,
7 comprising: administering an effective amount of an inhibitor compound to the animal.
8 In a particular embodiment the method is directed to inhibiting mucin overproduction in
9 the airway of an animal by administering an effective amount of an inhibitor compound
10 to target airway cells. An inhibitor compound is identified as described herein.
11 Typically, the inhibitor compound can, at a concentration of 500 μM or less, inhibit at
12 least 10% of total mucin production from cultured epithelial cells capable of mucomone
13 induced mucin production, preferably HM3 or HCIH292 cells, cultured under conditions
14 conducive to mucin production and in the presence of a mucomone, compared to
15 untreated cells. Preferably, the compound inhibits at least 30% and most preferably at
16 least 70% total mucin production at concentrations of preferably 50 μM or less, more
17 preferably 10 μM or less, even more preferably 1 μM or less, and most preferably 0.1 μM
18 or less.

19 Compositions for aerosol and enteral, especially oral, and for parenteral
20 administration are especially preferred. The compositions comprise an inhibitor of
21 mucomone induced mucin production alone or, preferably, together with a
22 pharmaceutically acceptable carrier. The dosage of the inhibitor depends upon the
23 disease to be treated and upon the species, its age, weight and individual condition, and
24 also upon the mode of administration.

25 Preferred is a pharmaceutical composition suitable for administration to a warm-
26 blooded animal, especially a human, suffering from a medical condition described herein,
27 for example CF, chronic bronchitis, bronchial asthma or bronchial pneumonia,
28 comprising an inhibitor compound described herein, or a salt thereof when salt-forming
29 groups are present, in an amount effective for the inhibition of the mucomone induced
30 mucin production, together with at least one pharmaceutically acceptable carrier.

31 Preferably, the compounds of the invention are formulated for pulmonary

1 administration. One such method of administration involves the aerosolization of a
2 solution containing, preferably, an aqueous-soluble compound of the invention. Aerosol
3 compositions can alternatively include the active compound packaged in reverse micelles
4 or liposomes. Pharmaceutical compositions suitable for such a method of administration
5 can additionally include aerosol propellants and a surfactant. Examples of small
6 compounds administered by this method can be found in U.S. Patent Nos. 5,364,615,
7 5,292,499, and 5,238,683, which are herein incorporated by reference. Both
8 phospholipid and nonconventional liposomes are rapidly becoming accepted as
9 pharmaceutical agents which improve the therapeutic value of a wide variety of
10 compounds (*Cancer Res.* 43:4730 (1983)) and can be applied to tyrosine kinase inhibitors
11 identified by methods of the present invention.

12 Compounds with poor solubility in aqueous systems require formulation by using
13 solubilizing agents such as ionic surfactants, cholates, polyethylene glycol (PEG),
14 ethanol, or other agents which may have undesirable effects when used for inhalation. In
15 addition, a treatment requiring successful delivery into alveoli of the lower pulmonary
16 region may preclude from the formulation the use of certain irritants such as
17 chlorofluorocarbons and should involve a minimum number of required doses.
18 Alternatively, to avoid such limitations, liposomes or hydrophobic particles can be used.
19 An inhalation formulation providing for a sustained release of such a compound using
20 aerosol droplet particles approximately 1-2.1 μ in size, preferably less than 1 μ , would
21 satisfy these special needs.

22 Small particle aerosol liposomes and liposome-drug combinations for medical use
23 have been previously disclosed in EP 87309854.5, which is herein incorporated by
24 reference. Conventional liposomal formulations sometimes have an uncontrollable and
25 fast release rate and frequently have larger particle sizes than are useful for directing a
26 drug to alveoli. However, nonconventional liposomes, which are formed solely by
27 cholesterol and cholesterol derivatives, or alternatively amphipathic lipid components,
28 have been used successfully with controllable sustained release, improved solubility, high
29 encapsulation, absence of need for multiple dosing, and extended stability. Suitable
30 liposomal formulations for sustained release of such compounds include sodium
31 cholesterol sulfate:cholesterol:compound % molar ratios of 55:40:5; 50:40:10; 53:37:9,

1 and most preferably 50:40:10 (U.S. Patent No. 5,049,389), which is hereby incorporated
2 by reference.

3 The pharmaceutical compositions comprise from approximately 5% to
4 approximately 95% active ingredient, dosage forms in single dose form preferably
5 comprising from approximately 20% to approximately 90% active ingredient and dosage
6 forms that are not in single dose form preferably comprising from approximately 5% to
7 approximately 20% active ingredient Unit dose forms, such as dragées, tablets or
8 capsules, comprise from approximately 0.05 g to approximately 1.0 g of active
9 ingredient.

10 The pharmaceutical compositions of this invention are prepared in a manner
11 known per se, for example by means of conventional mixing, granulating, confectioning,
12 dissolving or lyophilizing processes. For example, pharmaceutical compositions for oral
13 use can be obtained by combining the active ingredient with one or more solid carriers,
14 optionally granulating a resulting mixture, and, if desired, processing the mixture or
15 granules, if appropriate with the addition of additional excipients, to form tablets or
16 dragée cores.

17 Suitable carriers include fillers, such as sugars, for example lactose, saccharose,
18 mannitol or sorbitol, cellulose preparations and/or calcium phosphates, for example
19 tricalcium phosphate or calcium hydrogen phosphate, also binders, such as starches, for
20 example corn, wheat, rice or potato starch, methylcellulose,
21 hydroxypropylmethylcellulose, sodium carboxymethylcellulose and/or
22 polyvinylpyrrolidone, and/or, if desired, disintegrators, such as the above-mentioned
23 starches, also carboxymethyl starch, cross-linked polyvinylpyrrolidone, alginic acid or a
24 salt thereof, such as sodium alginate. Additional excipients include flow conditioners and
25 lubricants, for example silicic acid, talc, stearic acid or salts thereof, such as magnesium
26 or calcium stearate, and/or polyethylene glycol, or derivatives thereof.

27 Dragée cores can be provided with suitable, optionally enteric, coatings, there
28 being used inter alia concentrated sugar solutions which may contain gum arabic, talc,
29 polyvinylpyrrolidone, polyethylene glycol and/or titanium dioxide, or coating solutions in
30 suitable organic solvents or solvent mixtures, or, for the production of enteric coatings,
31 solutions of suitable cellulose preparations, such as acetyl-cellulose phthalate or

1 hydroxypropylmethylcellulose phthalate. Colourings or pigments may be added to the
2 tablets or dragée coatings, for example for identification purposes or to indicate different
3 doses of active ingredient.

4 Orally administrable pharmaceutical compositions also include dry-filled capsules
5 consisting of gelatin, and also soft, scaled capsules consisting of gelatin and a plasticizer,
6 such as glycerol or sorbitol. The dry-filled capsules may contain the active ingredient in
7 the form of granules, for example in admixture with fillers, such as corn starch, binders
8 and/or gildants, such as talc or magnesium stearate, and optionally stabilizers. In soft
9 capsules, the active ingredient is preferably dissolved or suspended in suitable liquid
10 excipients, such as fatty oils, paraffin oil, liquid polyethylene glycols or fatty acid esters
11 of ethylene or propylene glycol, to which stabilizers and detergents, for example of the
12 polyoxethylen-sorbitan fatty acid ester type, may also be added.

13 Other oral dosage forms are, for example, syrups prepared in customary manner
14 which comprise the active ingredient, for example, in suspended form and in a
15 concentration of about 5% to 20%, preferably about 10%, or in a similar concentration
16 that provides a suitable single dose, for example, when administered in measures of 5 or
17 10 ml. Also suitable are, for example, powdered or liquid concentrates for the
18 preparation of shakes, for example in milk. Such concentrates may also be packaged in
19 single dose quantities.

20 For parenteral administration compositions include suitable aqueous solutions of
21 an active ingredient in water-soluble form, for example in the form of a water-soluble
22 salt, or aqueous injection suspensions that contain viscosity-increasing substances, for
23 example sodium carboxymethylcellulose, sorbitol and/or dextran, and, if desired,
24 stabilizers. The active ingredient, optionally together with excipients, can also be in the
25 form of a lyophilisate and can be made into a solution prior to parenteral administration
26 by the addition of suitable solvents. Solutions such as those that are used, for example,
27 for parenteral administration can also be used as infusion solutions.

28 The invention relates also to a method of treating pathological conditions
29 associated with inappropriate mucin production and responsive to the inhibition of mucin
30 production. The compounds of this invention can be administered prophylactically or
31 therapeutically, preferably in an amount effective against the said diseases, to a warm-

1 blooded animal, for example a human, requiring such treatment, the compounds
2 preferably being used in the form of pharmaceutical compositions. In the case of an
3 individual having a body weight of about 70 kg the daily dose administered is from
4 approximately 1 mg to approximately 5000 mg, preferably from approximately 20 mg to
5 approximately 500 mg, of a compound of this invention.

6 The following examples are offered by way of illustration and not by way of
7 limitation. Variation and alternate embodiments will be apparent to those of skill in the
8 art.

9

10 EXAMPLES

11 Identification of the 5' MUC-5AC regulatory region

12 *Cell Culture* - The human lung epithelial carcinoma cell line NCIH292 was grown
13 in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Gibco).
14 In some experiments, cells were exposed for 6 h to *P. aeruginosa* to increase their
15 content of MUC 5AC mRNA.

16

17 *cDNA Synthesis and 5' RACE-PCR* - Two sources were used to prepare RNA for
18 cDNA synthesis, *P. aeruginosa* -exposed NCIH292 cells and human stomach tissue. The
19 sequences of the primers used for PCR are shown in Table 1. The RNA was prepared by
20 the method of Chomczynski and Sachi ((1987) *Anal. Biochem.* 162:156-159). Total
21 RNA (3 µg) was used to generate double-stranded (ds) cDNA using the Marathon cDNA
22 Amplification kit (Clontech). The ds cDNA was ligated with the Marathon cDNA
23 adaptor and purified on a chromaspin +TE-1000 column (Clontech) in a total volume of
24 100 µl. 5' RACE (Rapid Amplification of cDNA Ends, Frohman, (1991) *Meth. Enzymol.*
25 218:340-362) was performed using the ds cDNA as template with one HGM-1 gene-
26 specific primer (GM1) and the adaptor primer AP1 or AP2. Additional gene-specific
27 primers (GM5, GM9, GM9G, and GM9H) were generated based on the sequences of
28 progressively amplified 5' RACE products.

Table 1

Name	Sequence	Location
5' RACE PCR		
GM1	GGCCGTGGAGGATGCTGCACTGCTTCT (SEQ ID No. 3)	3348/3322
GM5(a)	GATGAAGCCAACACTGCAGGTGATGTCCC (SEQ ID No. 4)	2255/2227
GM9	AGAAGTGGGCACCTCCAAAGCACAGAGC (SEQ ID No. 5)	1374/1348
GM9G	AAGTCCCCACAGAGCCCACAGGTCTTG (SEQ ID No. 6)	688/662
GM9H(b)	TGTGTGCCGGGTGCAGGCCAGAGCGAGAG (SEQ ID No. 7)	119/90
AP1	CCATCCTAATACGACTCACTATAGGGC (SEQ ID No. 8)	Adapter
AP2	ACTCACTATAGGGCTCGAGCGGC (SEQ ID No. 9)	Adapter
Probes for Northern and genomic library screening		
NP3a 5'	TGCTATTATGCCCTGTGTAGCCAGGACTGCC (SEQ ID No. 10)	580/610(c)
NP3a 3'	TCACAGCCGGGTACGCGTTGGCACAAGTGGG (SEQ ID No. 11)	871/841(c)
TER	GGACAGGGCACTCTCCCCGCCGTCCAC (SEQ ID No. 12)	17/44
GM5	GATGAAGCCAACACTGCAGGTGATGTCCC (SEQ ID No. 13)	2255/2227
GM9	AGAAGTGGGCACCTCCAAAGCACAGAGC (SEQ ID No. 14)	1374/1348
GM2.6	GGCACGCGTGACACCAAATACGCCAACAAGACCTGTCCC (SEQ ID No. 15)	639/674
RNase protection and primer extension		
RPA-T7	GGATCCTAATACGACTCACTATAGGGAGGTATGCCGGGTGCAGGCCA (SEQ ID No. 16)	113/100
RPA-5'	GTGAGCACCCACTGTTTACTTGG (SEQ ID No. 17)	-123/-101
GM9H	TGTGTGCCGGGTGCAGGCCAGAGCGAGAG (SEQ ID No. 18)	119/90
Genomic walking		
GM9H5	CTTCCTCCGGCCAACACTCATTGTGTGGAC (SEQ ID No. 19)	68/39
AP1	GTAATACGACTCACTATAGGGC (SEQ ID No. 20)	Adapter
AP2	ACTATAGGGCACGCGCTGGT (SEQ ID No. 21)	Adapter

(a) Also used for northern

(b) Also used for primer extension

(c) Numbered according to Klomp, et al. (1995)

1 In the first round of 5' RACE-PCR, an HGM-1-specific primer (GM1) and an
2 adaptor primer (AP1) were used. This amplification yielded a 900 bp PCR fragment.
3 Sequence data showed that this fragment was the 5' extension of human gastric mucin
4 (HGM) (Klomp, et al. (1995), *Biochem. J.* 308:831-8 and was >65% similar to the MUC
5 2 D-domain 3 just 5' to the central repeat region. Primer GM5 was designed based on the
6 5' end of this fragment and was used in a second round of 5' RACE-PCR. This
7 generated an 1100 bp PCR fragment whose 5' end was used to design primer GM9.
8 When used in a third round of 5' RACE-PCR, GM9 generated a 700 bp fragment. Primer
9 GM9G was designed based on the 5' end of this fragment and was used in a fourth round
10 of RACE-PCR to generate a 600 bp fragment. Primer GM9H, 103 bp downstream of the
11 5' end of the fourth round RACE-PCR product, was used in a fifth round of RACE PCR
12 and generated a 110 bp product. Repeated efforts to generate larger products with primer
13 GM9H from both gastric tissue and NCIH292 (airway) cell cDNA yielded PCR products
14 with identical sequence that were ~100 bp in length. This suggested that GM9H was
15 approximately 100 bp from the 5' end of the mRNA as processed in both gastric tissue
16 and NCIH292 cells.

17 The overall cDNA sequence obtained by 5' RACE is shown in Figures 5 and is
18 about 3.3 KB (SEQ ID No. 2). There is an open reading frame (ORF) of 3300
19 nucleotides, 290 of which directly overlap and are in frame with those encoding human
20 gastric mucin. At +48 is an ATG codon embedded in a Kozak consensus sequence. This
21 is a putative translation start site. Following this is a secretory protein signal sequence.
22 The entire ORF encodes 1100 amino acids. The nucleotide sequence is approximately
23 65% similar to the MUC 2 amino terminal sequence. No tandem repeat sequence is
24 present, but there are three cysteine-rich domains (D1--D3) in which the cysteine
25 positions correspond almost exactly to those in the amino terminal of human MUC 2.

26 *Northern blot analysis of tissue distribution of RACE-PCR product cognate*
27 *RNA-* Total RNA was extracted from human tissues and cultured cells according to
28 previously described methods (Chomczynski and Sachi (1987) *Anal. Biochem.* 162:156-
29 159. RNA samples (20 µg) were separated on 1.0 % agarose gels containing 2.2 M
30 formaldehyde and then transferred to a positively charged nylon membrane (Gene
31 Screen, NEN, Dupont). cDNA probes were labeled with α -³²P dCTP using a BRL

1 random primer labeling kit. For the MUC 5AC 3' end, probes were amplified using
2 primers NP3a 5' and NP3a 5'; for the new sequence MUC 5AC-5' RP, probes were
3 amplified using primers TER and GM9. Labeled probe was added to 10 ml of
4 hybridization buffer containing 50% formamide, 10% dextran sulfate, 0.2% Denhardt's,
5 50 mM TRIS-HCl, pH 7.5, 1 M NaCl and 0.1% sodium pyrophosphate to give a
6 concentration of $2-5 \times 10^6$ cpm/ml. Membrane hybridization and washing were
7 performed using conditions described previously (Ohmori, et al. (1994) *J. Biol. Chem.*
8 269:17833-40).

9 Identical hybridization patterns were obtained when blots were probed with a
10 probe from the C-terminal cDNA, NP3a, (Meerzaman, et al. (1994), *J. Biol. Chem.*
11 269:12932-12939) or with a probe from the newly cloned N-terminal sequence.

12 *DNA Sequencing* - Fragments amplified by RACE-PCR were purified by low-
13 melting point agarose gel electrophoresis, cut with appropriate restriction enzymes and
14 cloned into pBluescript II SK⁻ (Stratagene) or sequenced directly. *E. coli* (SURE strain,
15 Stratagene) were transformed with plasmids containing these fragments. Transformants
16 were grown at 37°C or 30°C. Both sense and anti-sense strands were sequenced.
17 Sequencing reactions were carried out using SequiTherm Long-Read Cycle Sequencing
18 kits (Epicentre Technologies) and Thermo Sequenase Fluorescent Labeled Primer Cycle
19 Sequencing kits (Amersham Life Science) with the IRD41 (Li-cor) labeled primers.
20 Sequence data were assembled by Lasergene software (DNASTar). Homology and
21 transcription factor binding site searches were performed using MatInspector release 2.1
22 and Transcription Element Search Software (TESS, University of Pennsylvania) and
23 MacVector software (IBI).

24 *Chromosome localization of PCR-amplified DNA fragments* - Two
25 mouse/human hybrid cell line DNA panels were purchased from Bios. Cell line 1049
26 contained human chromosomes 5 and 11. Cell line 1079 contained human chromosomes
27 2 and 5. DNA from each cell line was used as a PCR template with RACE product
28 primers to determine the chromosomal location of RACE products. MUC 5AC- 5'
29 primers amplified a product from mouse-human hybrid cell line 1049, but not from cell
30 line 1079. As both cell lines contained DNA from chromosome 5 but only 1049
31 contained DNA from chromosome 11, the results clearly show that the RACE product

1 MUC 5AC-5'RP maps to chromosome 11.

2 *Primer extension analysis of transcription start site* - When progressive 5' RACE
3 reactions could no longer amplify additional sequence from either the stomach tissue or
4 airway cell (NCIH292) cDNA templates, primer extension using primer GM9H
5 (approximately 100 bp from the putative 5' end of the mRNA) was carried out to confirm
6 that the transcription start site had been reached. Primer extension was done using the
7 Promega AMV reverse transcriptase (AMVRT) primer extension system. Briefly, 0.1
8 pmole of ³²P end labeled primer GM9H was incubated with 5 µl (40-50 µg) total RNA
9 from tissue or cells and 5 µl of 2 x PE buffer at 58°C for 20 min. After cooling to room
10 temperature, 9 µl of a master mix containing 2 x PE buffer, 6.25 mM sodium
11 pyrophosphate and 1 µl AMV RT was added to each sample. After a 30 min. incubation
12 at 42°C, the samples were diluted with 20 µl loading dye, denatured by heating for 10
13 min at 90°C and run on a 6% acrylamide, 7M urea, TBE gel, along with sequencing
14 ladder and size markers.

15 The primer extension reaction yielded a product of 114 bp when RNA from
16 stomach tissue or airway cells was used as a template supporting the view suggested by
17 RACE-PCR that the transcription start site was approximately 100 bp upstream of
18 primer GM9H.

19 *RNase protection analysis of transcription start site* - To confirm transcription
20 start site location as determined by RACE-PCR and primer extension assays, RNase
21 protection assays were performed. The labeled RNA probe required for this assay was
22 generated from a PCR product designed to incorporate the T7 promoter. This PCR
23 fragment was amplified from a 12 kb genomic clone (7"A) derived from screening a
24 human genomic library in the Lambda FIX II vector (Stratagene) and was known from
25 sequencing data to contain the putative exon I of MUC 5AC. The library was screened
26 with a probe generated from PCR of a 5' RACE product with primers GM9 and GM2.6
27 using methods described in Ohmori, et al., (1994). The primers used to generate the
28 RNA probe template from the genomic clone were RPA-T7 containing sequence from
29 exon I and the T7 promoter and primer RPA-5' containing upstream genomic sequence
30 (see Table 1). This enabled the generation of a high specific activity ³²P-UTP labeled
31 RNA probe using T7 RNA polymerase. For the assay, 50 µg of total RNA was

1 hybridized with 5×10^5 cpm of probe overnight at 42°C. The RNA:RNA template was
2 digested for 15 min. at room temperature with 0.5 units of RNase A and 20 units of
3 RNase T1, precipitated and run on a 6% polyacrylamide/urea sequencing gel with a
4 sequencing ladder for size determination.

5 A total of three RNA samples were examined. These samples were taken from
6 gastric tissue, colon carcinoma cells (HM3) and lung carcinoma cells (NCIH292). RNA
7 from each sample protected the same three probe fragments, indicating putative start sites
8 at one, six and eight bp upstream of the start site predicted by primer extension. The start
9 site predicted by computer program NNPP (promoter prediction by neural network,
10 Lawrence Berkeley National Laboratory, Human Genome Center) was at 4 bp upstream
11 of the site indicated by primer extension. As it fell approximately in the middle of the
12 range of possible start sites, the computer-predicted start site was designated as +1.

13 *5' genomic DNA walking* - Genomic DNA was amplified from DNA provided in
14 the Human PromoterFinder™ DNA Walking kit (Clontech) according to instructions
15 provided by the manufacturer. Long sequence amplifications were carried out with the
16 LA PCR kit (TaKaRa) and High Fidelity Expand PCR kit (Boehringer Mannheim) using
17 primers GM9H5' and adaptor primers AP1 and AP2.

18 These amplifications yielded an approximately 4KB genomic DNA fragment
19 containing 3753 nucleotides 5' to the MUC 5AC transcription start site identified above.
20 The sequence is shown in Figure 1A and B (SEQ ID No. 1). The sequence from -300 to
21 +1 as well as downstream sequence through exon 1 (+1 to +120) has been confirmed by
22 sequencing a subclone of genomic clone 7''A. The upstream sequence contains a TATA
23 box at -28/-31, further supporting the view that the RACE-PCR product MUC 5AC-5'RP
24 is at the 5' end of the mRNA and that the designated transcription start site, +1 is
25 accurate. Present in the putative promoter region are NF kappa B sites (at -216 through -
26 228 and -950 through -959), Sp-1 sites (at -149 through -154 and -76 through -84), GRE
27 site (at -311 through -316 and -1253 through -1258), AP-2 sites (at -458 through -463 and
28 -1098 through -1103), PEA site (at -930 through -935) and CACCC box (at -65 through -
29 70) sites.

30

31

1 Example 2 Construction of Reporter Gene Construct

2 A DNA fragment extending from -3752bp to +68 bp was cloned into the
3 MluI/SmaI site of the pGL3 vector (Promega). pGL3 contains a luciferase gene
4 downstream from the SmaI site. This construct, referred to as M4-2, was deposited with
5 the American Type Culture Collection as ATCC designation 98701. M4-2 was co-
6 transfected with pcDNA3, containing the neomycin resistance gene, into the epithelial
7 cell line HM3. G418-selected colonies were pooled, expanded, and used in luciferase
8 assays.

9 A DNA fragment, synthesized by PCR, extending from -614 bp to +68 bp was
10 cloned into pGL3 in a similar fashion. This construct is referred to as -614 construct.

11

12 Example 3 Activation of Reporter Gene Construct by Environmental Tobacco Smoke

13 HM3 colon carcinoma cells (Kuan, et al. (1987) *Cancer Res.* 47:5715-5724) or
14 NCIH292 lung carcinoma cells (Levine, et al. (1995) *Am. J. Respir. Disease* 12:196-204)
15 were transfected with the 4.0Kb MUC 5AC reporter gene construct, M4-2, or the -614
16 construct from Example 2. Transfection was carried out essentially as described in
17 Felgner et al. (1987), *supra*. In brief, 2 μ l lipofectamine and 1 μ g DNA were added to
18 approximately 5×10^6 cells in 100 μ l of Opti-men (Gibco) and incubated at room
19 temperature for about 30 minutes. The mixture was then diluted to 1 ml and added to one
20 culture well of a 6 well plate. G418-selected colonies were pooled, expanded and used in
21 luciferase assays.

22 Environmental tobacco smoke was used as a mucomone. Culture medium DME-
23 F12 (Dulbecco's Modified Eagle's:Ham's F12 at 1:1) was exposed to tobacco smoke at
24 various concentrations, for 6 hours. Exposure of the medium was carried out in open
25 culture dishes in an enclosed container into which tobacco smoke produced by burning
26 cigarettes was introduced in a controlled manner. Smoke concentration was measured in
27 units of total suspended particles/ml (TSP/ml). Transfected cells were transferred into
28 the smoke-exposed medium (in 6-well plates, 5×10^5 cells per well) and incubated for
29 various times. The cells were then harvested, lysed and the luciferase activity determined
30 as described in Li et al. (1997).

31 Figure 2 shows the results of an experiment using HM3 cells transfected with the

1 4.0 Kb MUC 5AC construct, M4-2, and incubated in smoke-exposed medium for 6 hr.,
2 14 hr. or 21 hr. The medium was exposed to three concentrations of smoke: 8.5 TSP/ml,
3 17 TSP/ml and 34 TSP/ml. The control cells were treated in an identical manner but
4 were not exposed to smoke medium. The relative luciferase activity (RLA) was
5 measured in light units.

6 Relative luciferase activity increased with both time of exposure of the cells to
7 smoke medium and concentration of smoke used to prepare the smoke medium. At the
8 highest level of smoke concentration tested (34 TSP/ml) at 21 hr, the RLA was 8-9 fold
9 higher than the control level.

10 Figure 3 shows the results of an experiment using NCIH292 cells transfected with
11 the M4-2 construct and incubated in smoke-exposed medium for 6 hr and 14 hr with the
12 same three smoke concentrations as for the HM3 cells above.

13 NCIH292 cells transfected with the -614 construct also showed increased
14 luciferase activity after culture in smoke-exposed medium although the magnitude of the
15 increase was not as large as that of the M4-2 transfected cells.

16

17 Example 4 Inhibition of Mucomone-induced Reporter Gene Activation

18 Several possible inhibitors of mucomone-induced mucin production were tested
19 for their effect on the smoke induced luciferase activity described in Example 3.

20 Inhibitors tested included the inhibitors Genistein (Calbiochem), anti-MEK (PD98059;
21 Alessi et al. 1995), PKC-I (Bisindolymaleimide) and PKA-I (KT 5720, Calbiochem).

22 HM3 cells transfected with the 4.0 KB MUC 5AC construct, M4-2, were incubated in
23 smoke-exposed medium as described in Example 3. The inhibitors were added to the
24 cells at the concentrations indicated in Table 2 for various times before the cells were
25 transferred to the smoke medium. Additional inhibitor was added to the smoke medium
26 after the cells were transferred. The results are shown in Figure 4. For each inhibitor
27 tested, the RLA was lower in cells exposed to inhibitor than in cells not exposed to
28 inhibitor, indicating that the smoke-induced luciferase activity was inhibited.

1
2

Table 2

Inhibitor	Concentration	Times Before Addition of Smoke- Medium
Bisindolymaleimide	2.5 nM to 10 nM	1 hr
KT 5720	0.1 to 0.5 μ M	15 min - 1 hr
Genistein	30 μ M	30 min - 2 hr
PD98059	50-100 μ M	1 hr

3
4

Example 5 Activation of Reporter Gene Constructs containing a heterologous promoter.

Additional reporter gene constructs were made containing the MUC 5AC 5' regulatory region from -3752bp to -143bp ("long") or from -3752bp to -3535bp ("short") joined upstream of a minimal thymidine kinase (tk) promoter which was operably joined to a luciferase reporter gene (coding sequence). The tk promoter-luciferase construct was described in Li et al., Proc. Natl Acad. Sci USA, 95:5718 (1998). Exposure of the constructs to environmental tobacco smoke was carried out as described in Example 3. Both the long and the short MUC 5AC fragments conferred comparable smoke inducibility on the tk promoter of approximately 15-fold over control. Control constructs without the MUC 5AC fragments did not show inducibility upon exposure to smoke. These results suggest that one or more smoke response elements are present in the MUC 5AC 5' regulatory region between -3752bp and -3535bp (SEQ ID No. 1 from nucleotide 1 to 218).

18

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

21

1

2 The invention now being fully described, it will be apparent to one of ordinary
3 skill in the art that many changes and modifications can be made thereto without
4 departing from the spirit or scope of the appended claims.

1 WHAT IS CLAIMED IS:

2 1. An isolated polynucleotide molecule comprising at least 20 consecutive
3 nucleotides of the MUC 5AC 5' regulatory region (SEQ ID No:1), or a complement
4 thereof.

5
6 2. The polynucleotide molecule of Claim 1 comprising at least 50 consecutive
7 nucleotides of the MUC 5AC 5' regulatory region (SEQ ID No:1), or a complement
8 thereof.

9
10 3. The polynucleotide molecule of Claim 1 comprising at least 100 consecutive
11 nucleotides of the MUC 5AC 5' regulatory region (SEQ ID No:1), or a complement
12 thereof.

13
14 4. The isolated polynucleotide molecule of Claim 1, having the sequence of SEQ
15 ID No. 1, or the complement thereof.

16
17 5. The isolated polynucleotide molecule of Claim 1, selected from the group
18 consisting of a polynucleotide molecule having the sequence of SEQ ID No.: 1 from
19 nucleotide 1 to nucleotide 218, a polynucleotide molecule having the sequence of SEQ
20 ID No.: 1 from nucleotide 2392 to nucleotide 3752, and a polynucleotide molecule
21 having the sequence of SEQ ID No.: 1 from nucleotide 3139 to nucleotide 3752, and
22 complements thereof.

23
24 6. An isolated polynucleotide molecule comprising a mucomone response
25 element of the MUC 5AC 5' regulatory region.

26
27 7. The polynucleotide molecule of claim 6, wherein said mucomone response
28 element comprises at least 20 consecutive nucleotides from SEQ ID No:1.

29
30 8. The polynucleotide molecule of claim 7, wherein said mucomone response
31 element comprises a polynucleotide molecule selected from the group consisting of a

1 polynucleotide molecule having the sequence of SEQ ID No.: 1 from nucleotide 1 to
2 nucleotide 218, a polynucleotide molecule having the sequence of SEQ ID No.: 1 from
3 nucleotide 2392 to nucleotide 3752, and a polynucleotide molecule having the sequence
4 of SEQ ID No.: 1 from nucleotide 3139 to nucleotide 3752, and complements thereof.

5

6 9. A mucomone-inducible reporter gene construct comprising:
7 a mucomone response element from the 4KB MUC 5AC 5' regulatory region and
8 a responsive promoter, operably linked to a reporter gene.

9

10 10. The reporter gene construct of Claim 9, wherein said responsive promoter is a
11 mucin promoter.

12

13 11. The reporter gene construct of Claim 10, wherein said mucin promoter is a
14 MUC 5AC promoter.

15

16 12. The reporter gene construct of Claim 9, wherein said reporter gene is selected
17 from the group consisting of chloramphenicol acetyl transferase, beta-galactosidase, β -
18 glucuronidase, firefly luciferase, bacterial luciferase, alkaline phosphatase and green
19 fluorescent protein.

20

21 13. The reporter gene construct of Claim 9, wherein said mucomone response
22 element comprises at least 50 consecutive nucleotides of the MUC 5AC 5' regulatory
23 region (SEQ ID No:1).

24

25 14. The reporter gene construct of Claim 9, wherein said mucomone response
26 element comprises at least 100 consecutive nucleotides of the MUC 5AC 5' regulatory
27 region (SEQ ID No:1).

28

29 15. The reporter gene construct of Claim 9, wherein said construct is ATCC
30 98701.

31

1 16. The reporter gene construct of Claim 9, wherein said mucomone response
2 element comprises a polynucleotide molecule selected from the group consisting of a
3 polynucleotide molecule having the sequence of SEQ ID No.: 1 from nucleotide 1 to
4 nucleotide 218, a polynucleotide molecule having the sequence of SEQ ID No.: 1 from
5 nucleotide 2392 to nucleotide 3752, and a polynucleotide molecule having the sequence
6 of SEQ ID No.: 1 from nucleotide 3139 to nucleotide 3752, and complements thereof.

7
8 17. The reporter gene construct of claim 9, wherein said responsive promoter is a
9 thymidine kinase promoter.

10
11 18. An epithelial cell comprising the reporter gene construct of Claim 9.

12
13 19. The cell of Claim 18, wherein said reporter gene construct is stably
14 transfected into the chromosome of said cell.

15
16 20. A method for identifying an inhibitor compound useful for inhibiting mucin
17 production comprising:

18 (a) contacting cells comprising a mucomone-inducible reporter gene construct
19 with a mucomone, wherein said reporter gene construct comprises a mucomone response
20 element comprising at least 20 consecutive nucleotides of the MUC 5AC 5' regulatory
21 region (SEQ ID No:1) and a responsive promoter, operably linked to a reporter gene,

22 (b) contacting said cells with a test compound, and
23 comparing the reporter gene activity after performing steps (a) and (b) with the
24 reporter gene activity after performing only step (a).

25
26 21. The method of Claim 20, wherein said responsive promoter is a MUC
27 5AC promoter or a thymidine kinase promoter.

28
29 22. The method of Claim 20, wherein said mucomone response element
30 comprises a polynucleotide molecule selected from the group consisting of a
31 polynucleotide molecule having the sequence of SEQ ID No.: 1 from nucleotide 1 to

1 nucleotide 218, a polynucleotide molecule having the sequence of SEQ ID No.: 1 from
2 nucleotide 2392 to nucleotide 3752, and a polynucleotide molecule having the sequence
3 of SEQ ID No.: 1 from nucleotide 3139 to nucleotide 3752, and complements thereof.
4

5 23. The method of Claim 20, wherein said reporter gene is selected from the
6 group consisting of firefly luciferase, bacterial luciferase, chloramphenicol
7 acetyltransferase, β -galactosidase, β -glucuronidase, alkaline phosphatase and green
8 fluorescent protein.
9

10 24. The method of Claim 20, wherein the reporter gene activity determined
11 after performing only step (a) is at least two-fold higher than the reporter gene activity
12 determined after performing steps (a) and (b).
13

14 25. The method of Claim 20, wherein said mucomone is selected from the
15 group consisting of *Pseudomonas aeruginosa*-conditioned medium, *E. coli*-conditioned
16 medium, *Pseudomonas aeruginosa* lipopolysaccharide and environmental tobacco
17 smoke-conditioned medium.
18

19 26. The method of Claim 20, wherein said mucomone response element
20 comprises at least 50 consecutive nucleotides of the MUC 5AC 5' regulatory region (SEQ
21 ID No:1).
22

23 27. The method of Claim 20, wherein said mucomone response element
24 comprises at least 100 consecutive nucleotides of the MUC 5AC 5' regulatory region
25 (SEQ ID No:1).
26

27 28. A pharmaceutical composition for treatment of mucin overproduction in
28 the airway of an animal, comprising an effective amount of an inhibitor compound
29 identified by the method of Claim 20 and a pharmaceutically acceptable carrier.
30

31 29. A method for inhibiting mucin overproduction in the airway of an animal,

1 said method comprising: administering to said animal an effective amount of an inhibitor
2 identified by the method of Claim 20.

3

4 30. A method for identifying an inhibitor compound useful for inhibiting mucin
5 production induced by tobacco smoke comprising:

6 (a) contacting cells comprising a mucomone-inducible reporter gene construct
7 with tobacco smoke, wherein said reporter gene construct comprises a mucomone
8 response element comprising at least 20 consecutive nucleotides of the MUC 5AC 5'
9 regulatory region (SEQ ID No:1) and a responsive promoter, operably linked to a reporter
10 gene,

11 (b) contacting said cells with a test compound, and

12 comparing the reporter gene activity after performing steps (a) and (b) with the
13 reporter gene activity after performing only step (a).

14

15 31. The method of Claim 30, wherein said mucomone response element
16 comprises the sequence of SEQ ID No.: 1 from nucleotide 1 to nucleotide 218.

17 32. The method of Claim 30, wherein said responsive promoter is a mucin
18 promoter or a thymidine kinase promoter.

1 / 6

First Nucleotide is -3752

GGTCGACGGCCCCGGGCTGGTCTGGACCCCAGCAGCGGCCCTGGGTGACGTCTGGCTGAGGGAGG
AGAAAGCTGTGGCTGGGGCGGCAAGGCCTGGGTGGCCAGTTGGCCAGGTGCCCCGGGGCTTGGC
CCAGCCTCAGACACGCAGGGGGGCACTCCCCTCTGAGGGCCACGCTGGTGACTCAGACTGTTTCAG
AGGTCACGGTATGGACTGGGCCAGTGACTCAGGCCTGTCTCTGTTGGGGGCTGGACACTGACT
CACCCACTGCCTCCTGTCTATCTGAGGGCGTAAGGAGGGCAGGCCTTCAGGCACTCACATGCGG
CCCTGGCCAGGGTCCCGGTCACACCTGCAGACCCTCAAGCCCTTCCCTATGCCCCACTGACATA
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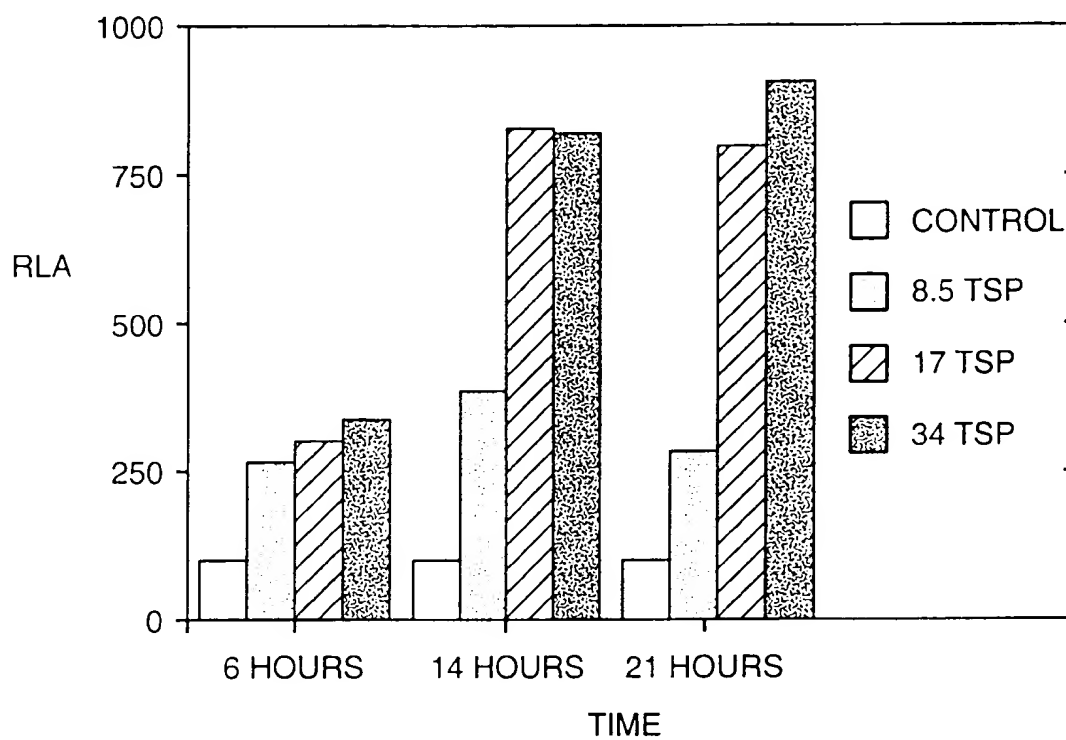
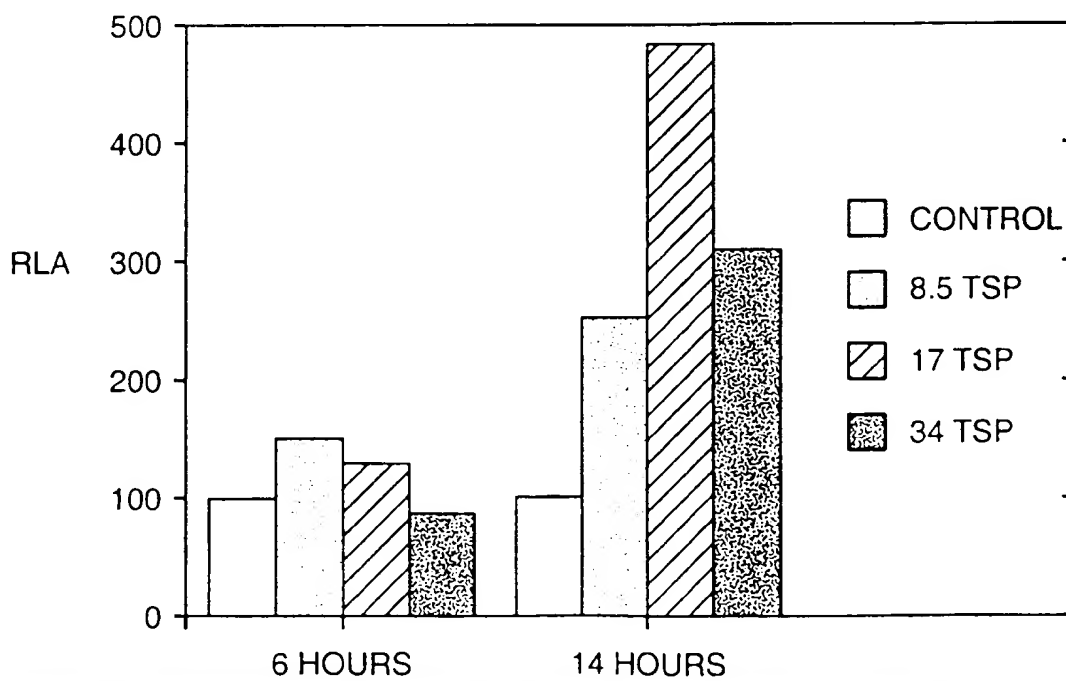
FIG. 1A

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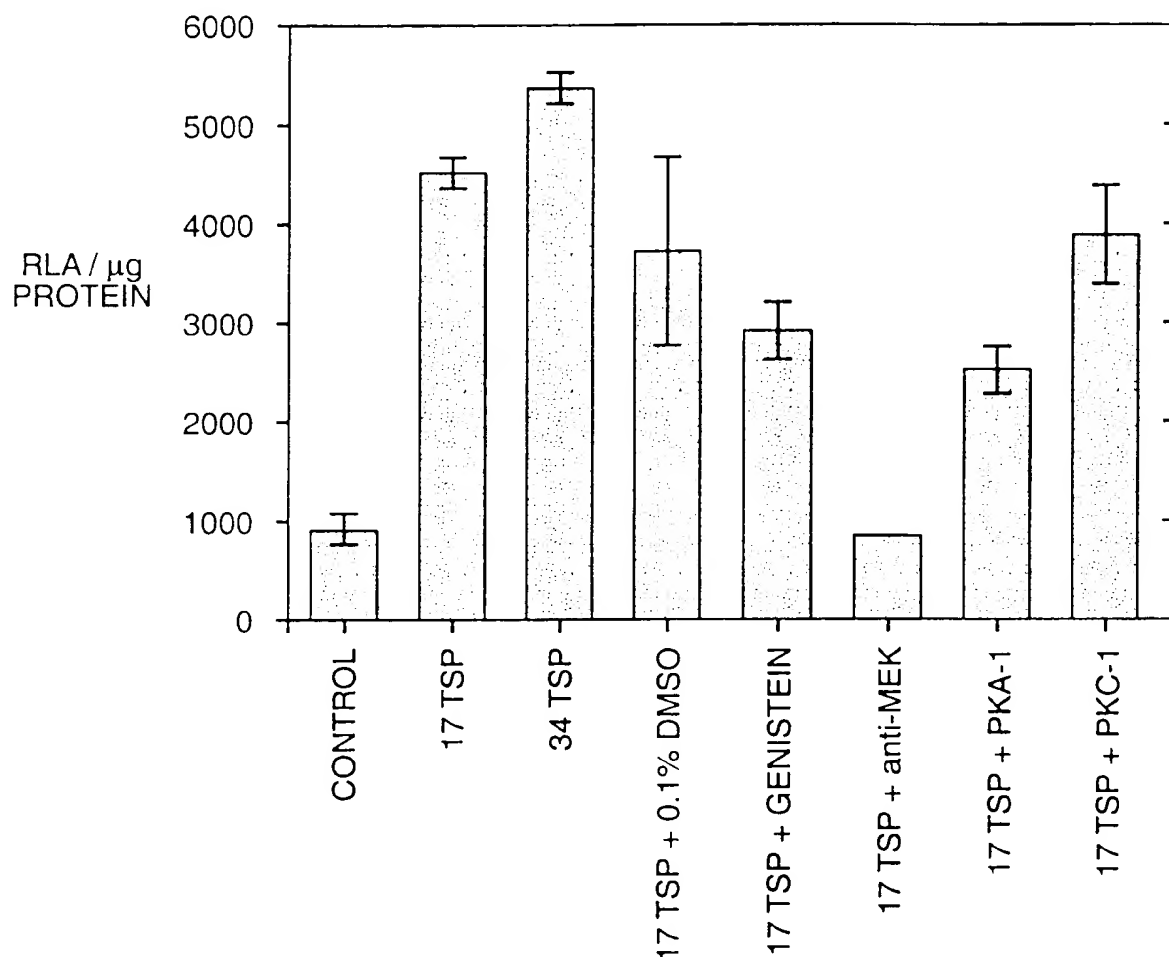
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FIG. 1B

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**FIG. 2**

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**FIG. 4**

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FIG. 5

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+1

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FIG. 5A

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FIG. 5B

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DAIZONG, LI
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GENSCH, ERIN

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tatctcag 428

361778 v1/PA

INTERNATIONAL SEARCH REPORT

International application No
PCT US99 03024

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) C07H 21/02, 21/04; C12N 5/00

US CL 536/23.1, 24.1, 24.2; 435/325

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U S 536/23.1, 24.1, 24.2; 435/325

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
noneElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)
STN (Medline, Biosis, Embase), APS, Derwent

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	LI, D. et al. Cloning of the Amino-terminal and 5'-Flanking Region of the Human <i>MUC5AC</i> Mucin Gene and Transcriptional Up-regulation by Bacterial Exoproducts. The Journal of Biological Chemistry. 20 March 1998, Vol. 273, No. 12, pages 6812-6820, see entire reference.	1-32
Y	WO 97/43643 A1 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 20 November 1997, see entire document.	1-32



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

20 APRIL 1999

Date of mailing of the international search report

14 MAY 1999

INTERNATIONAL SEARCH REPORT

International application No
PCT US99 03024

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
A	BOUCHER, J. C. et al. Mucoid <i>Pseudomonas aeruginosa</i> in Cystic Fibrosis: Characterization of <i>muc</i> Mutations in Clinical Isolates and Analysis of Clearance in a Mouse Model of Respiratory Infection. Infection and Immunity. September 1997, Vol. 65, No. 9, pages 3838-3846, see entire reference	1-32
Y	LI, J.D. et al. Transcriptional activation of mucin by <i>Pseudomonas aeruginosa</i> lipopolysaccharide in the pathogenesis of cystic fibrosis lung disease. Proc. Natl. Acad. Sci. USA. February 1997, Vol. 94, pages 967-972, see entire reference.	1-32